

# Ketogenic Diet and Ketone Bodies Enhance the Anticancer Effects of PD1 Blockade

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## Supplemental materials

### **Figure S1: Metabolomics profiling of distant tissues (liver, heart).**

Refer to Figure 2B, D. Heatmap of the non-supervised hierarchical clustering highlighting differences in the metabolic profiling of C57BL/6 mice fed normal diet (ND) versus ketogenic diet (KD) in heart (A) and liver (B) at day 12 post-start of KD versus ND. Each line represents one mouse metabolomics analysis.

### **Figure S2: Pharmacokinetics profiling of 3-hydroxybutyrate plasma concentrations during various diet interventions.**

Longitudinal follow up of plasma concentrations of 3HB monitored in MALDI-TOF following various nutritional interventions in C57BL/6 mice [tumor-free (A) or RET tumor-bearers (B)] and in BALB/c mice depicted as area under the curve (AUC) (C). One to two experiments for each setting containing 6 mice/group has been performed. The diet was allowed in continuous (Cont) or with the intermittent On/off scheduling.

**Figure S3: Metabolomics profiling of plasma when ketosis is blunted by the addition of sucrose.**

Refer to Figure 3D-F. Heatmap of log2-fold changes in the non-supervised hierarchical clustering highlighting differences in the metabolic profiling of RET tumor-bearing C57BL/6 mice fed ketogenic diet (KD) +/- 10% sucrose in drinking water. Each line represents one mouse metabolomics analysis.

**Figure S4: Ketogenic diet-induced changes in the intestinal taxonomic bacteria composition.**

A. Volcano plot segregating significant over-or under-represented OTUs in normal diet (ND) versus ketogenic diet (KD) fed mice with p values and fold changes. B. Relative abundances of *A. muciniphila*, *R. lactatiformans*, *C. asparagiforme*, *P. capillosu*, *Turicibacter sanguinis* and *Lactobacilli* spp. in KD versus ND fed mice at day 12 represented as bar graphs of means +SEM in 6 mice/groups. C. Id. as in Figure 5A but showing the ND group.

**Figure S5: Phenotyping of T cell splenocytes for inhibitory receptor expression .**

A-B. Flow cytometry determination of various surface markers (PD-1, CTLA-4 ,4-1BB, Tim-3, Lag-3) expressed in CD4+ (A) and CD8+ (B) T cell subsets at day 15 in the spleens of BALB/c mice subjected to dietary interventions and cICB therapy. The results from 2 experiments comprising 6 mice/group are depicted, each dot representing one spleen. C. Blood monocyte enumeration. D. Phenotype gating in CD11b<sup>+</sup>CD11c<sup>-</sup>/CD45<sup>+</sup> in naive and RET tumor bearers at day 5 after starting ND, KD or 3HB per os. Each dot represents one mouse. One experiment out of 2-4 yielding similar results is presented. D. Flow cytometry determination of the cell surface expression of PD-L1 by RET cell line after a 48 hours exposure to 3HB +/-

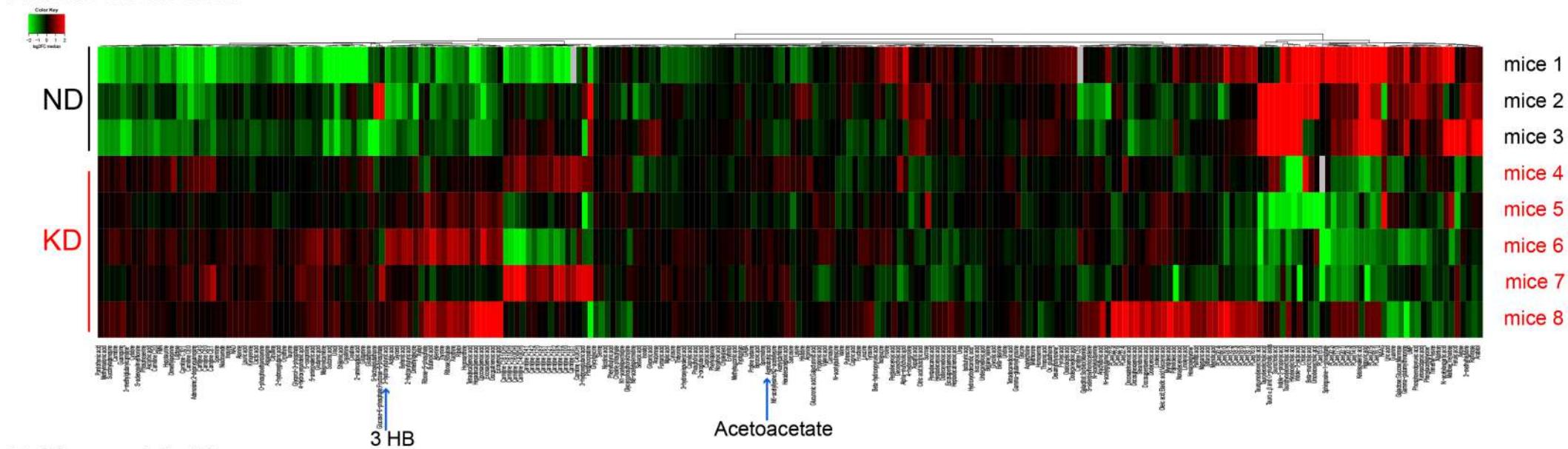
rIFNg. A representative overlay of MFI is depicted, out of three leading to similar results.

Statistics: Mann Whitney, Student's t-test. \*p<0.05, \*\*p<0.01, \*\*\*p<0.001.

**Figure S6: Experimental settings for figure 8.**

A-B. Orthotopic tumor model (RENCA-luciferase kidney cancer syngeneic of BALB/c (A)) and metastasis from lung cancer (TC-1-luciferase syngeneic of C57BL/6 mice (B)) were established 7 to 10 days prior to starting immunotherapy with anti-PD1 and/or anti-CTLA4 Abs. The diet interventions have been initiated either at the time of RENCA implantation or 9 days prior to iv injection of TC-1-luc. C. Assessment of memory responses in tumor free (cured) animals used from the experiments described in Figure 8 or naive animals as positive controls. Rechallenge with inoculation of 10 times the minimum tumorigenic dose of tumor cells.

### A. Heart metabolites



### B. Liver metabolites

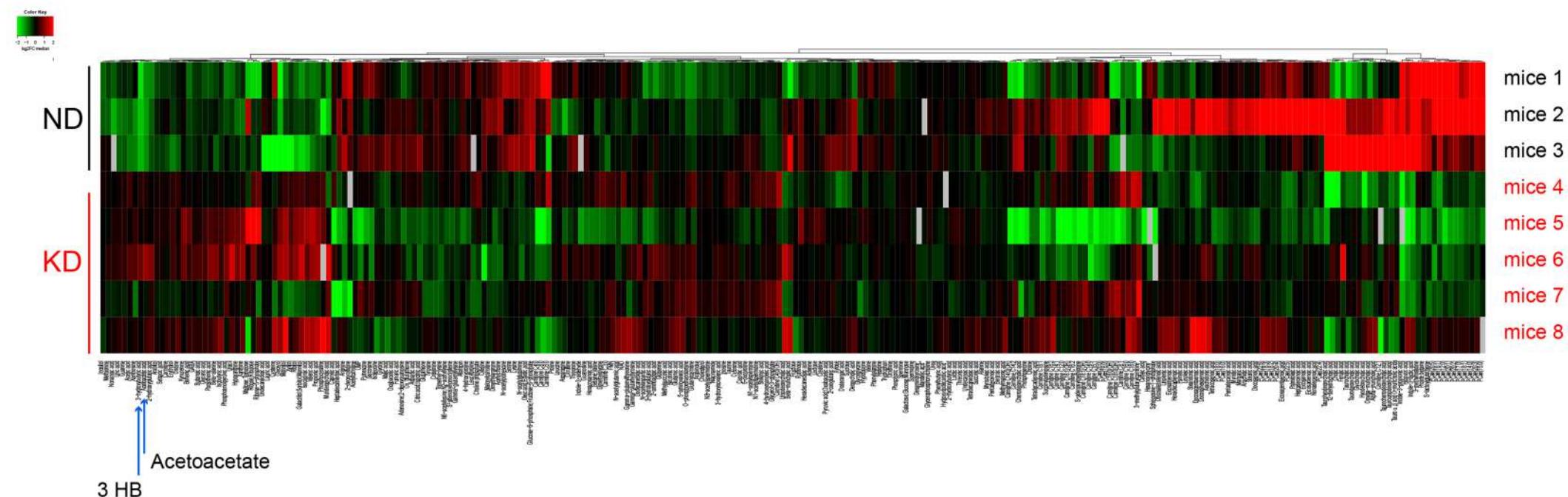


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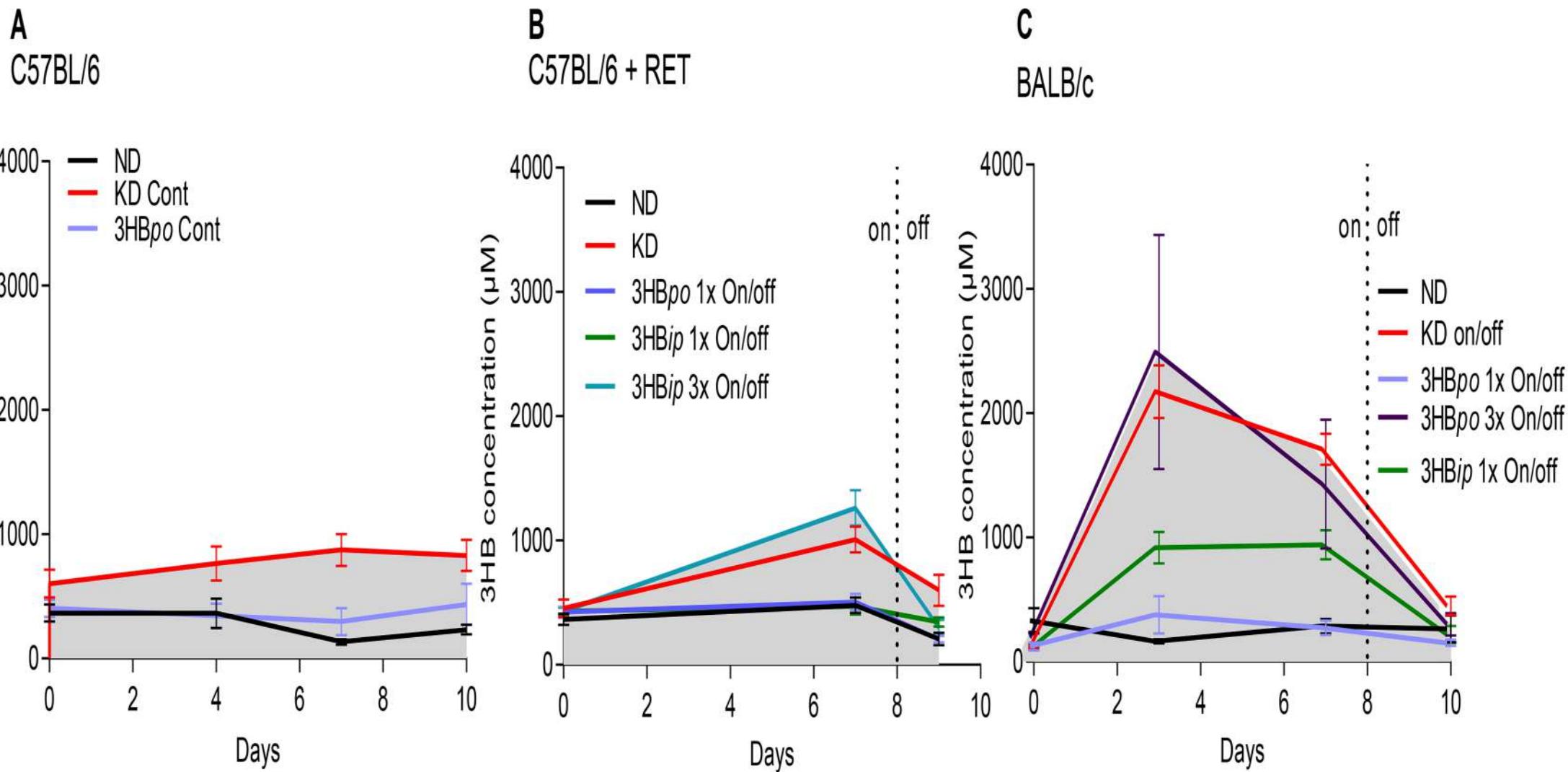


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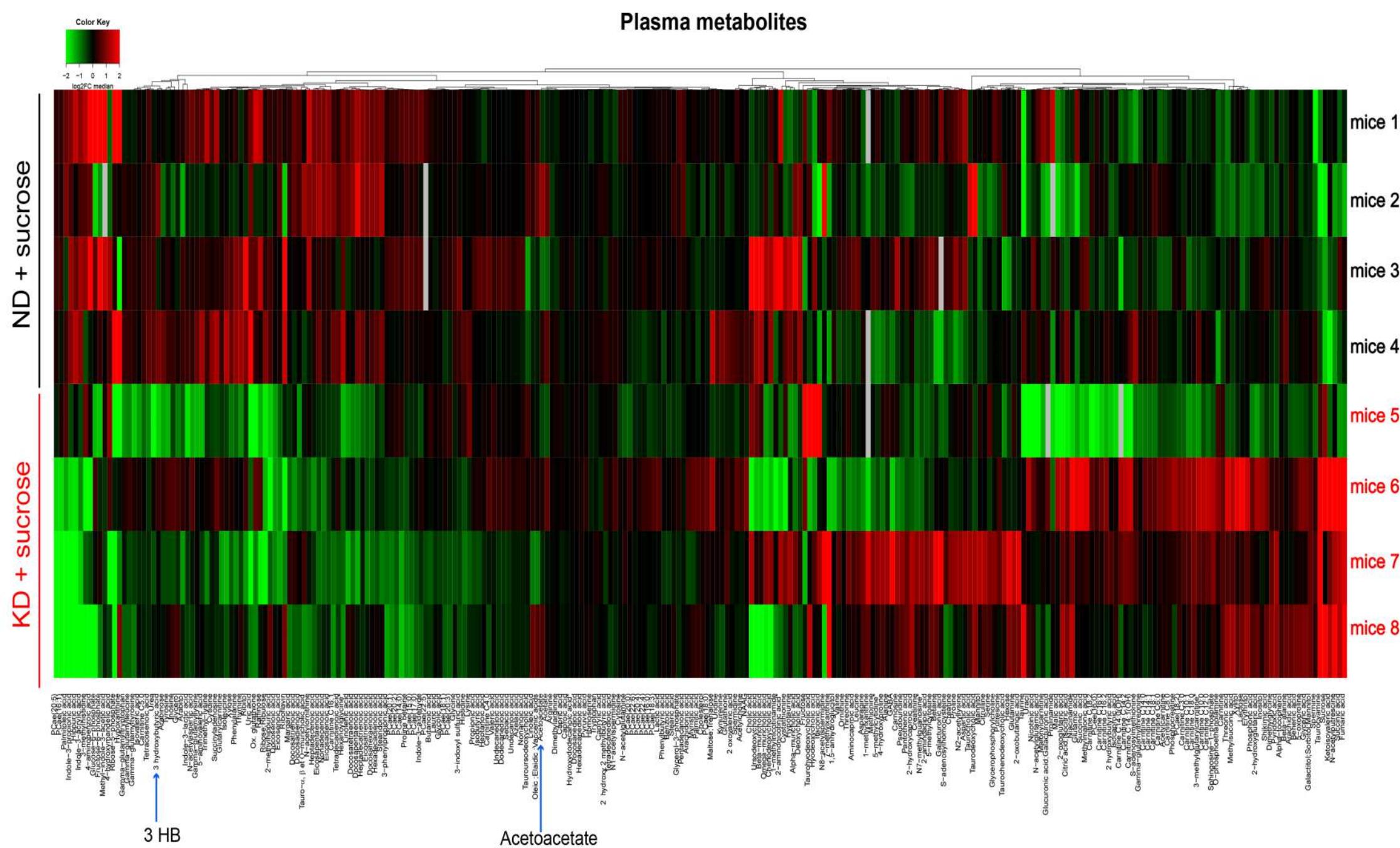


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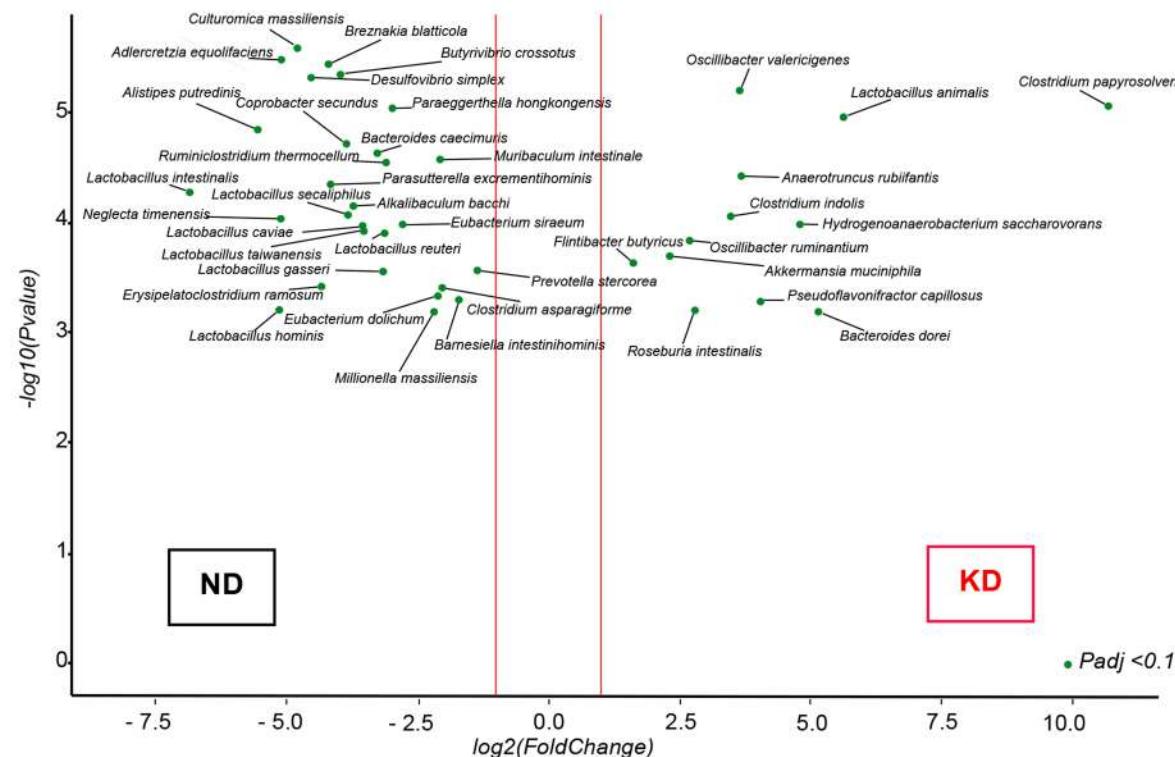
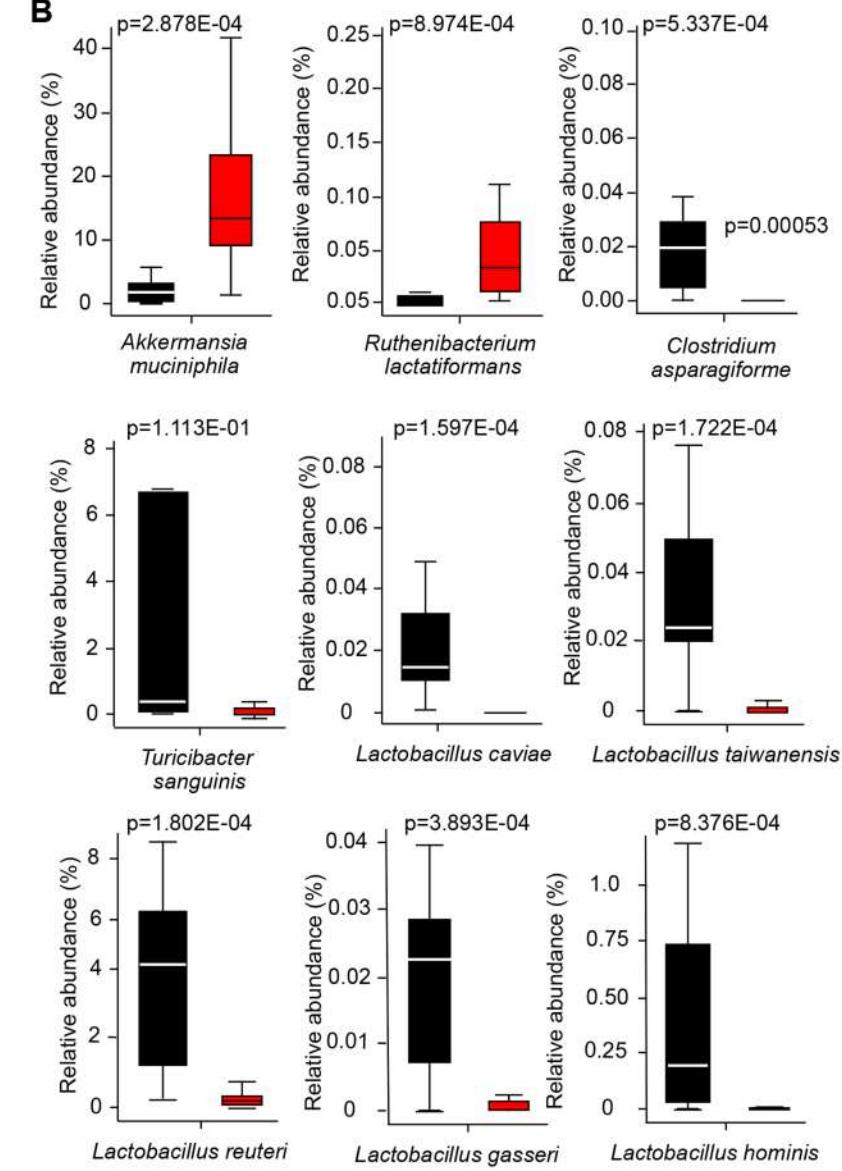
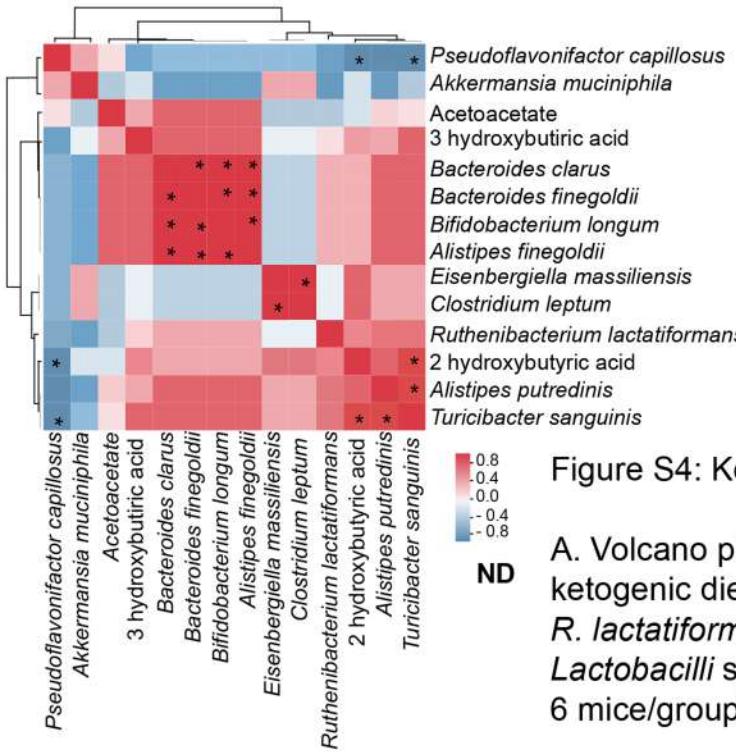
**A****B****C**

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■ ND  
■ KD

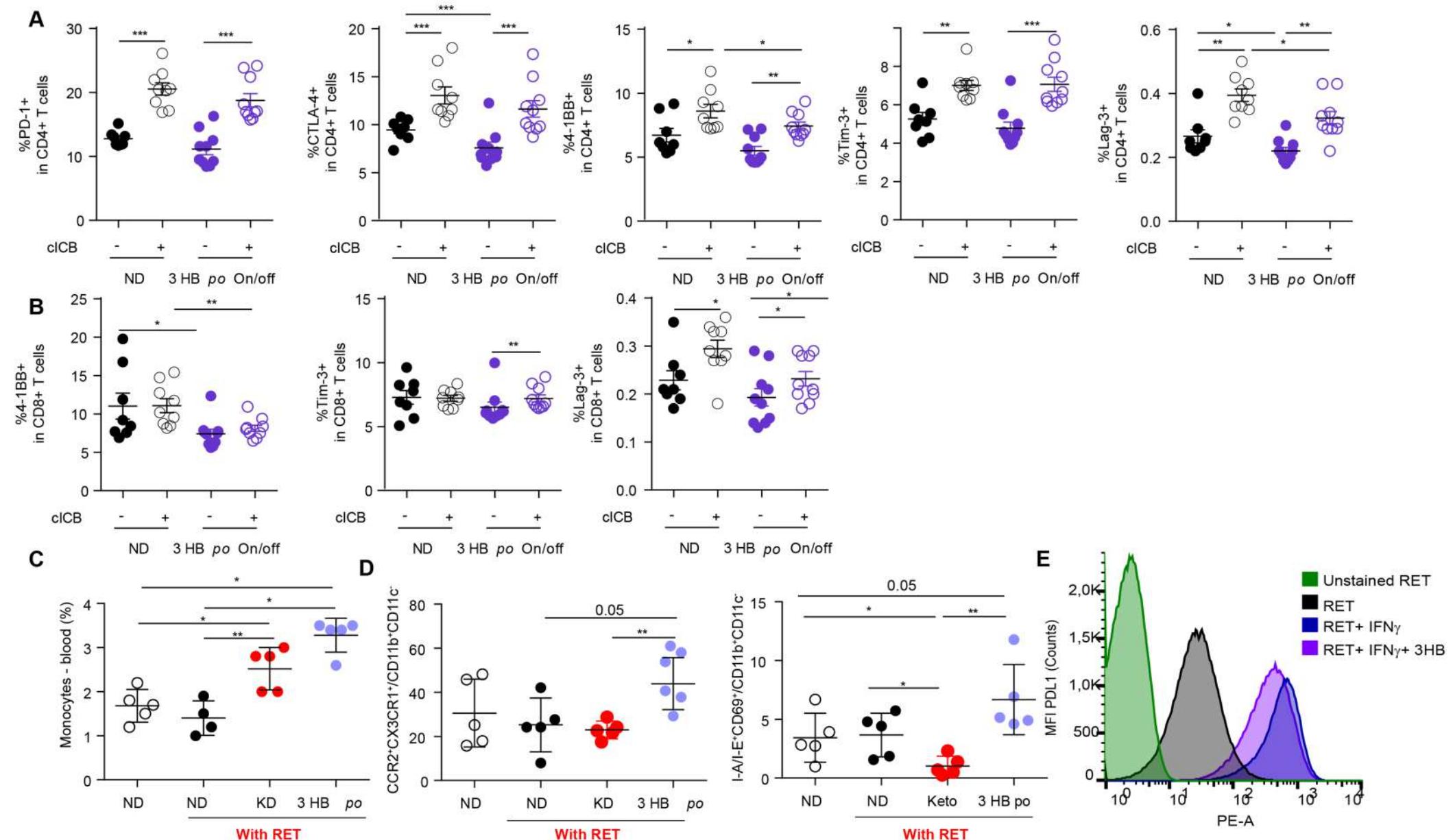


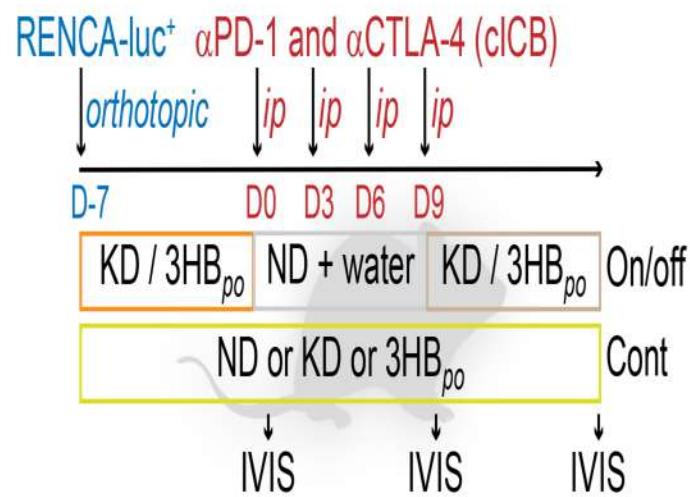
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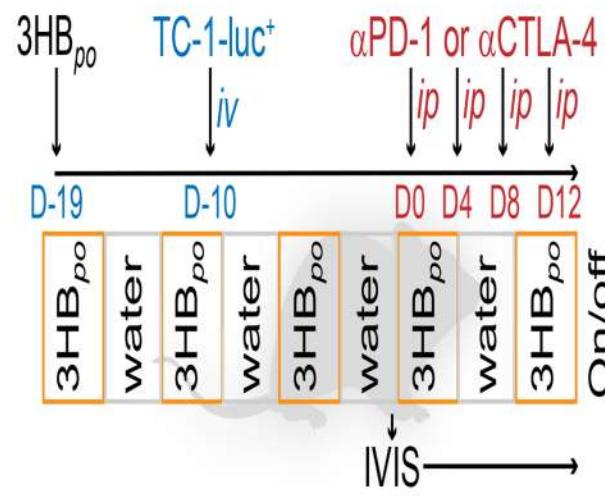
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A



B



C

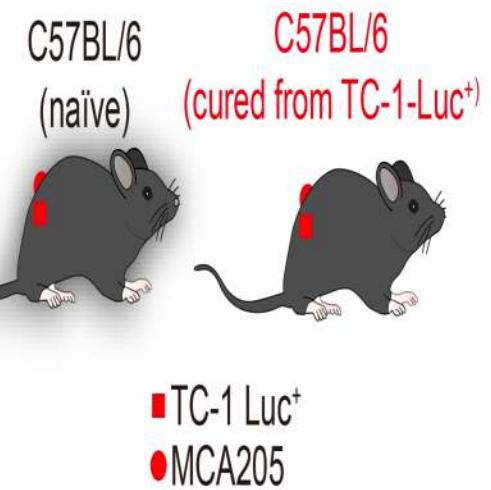


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**Supplemental table 1. List of metabolites differentially monitored in plasma of ketogenic diet-fed animals versus normal diet -fed littermates.**

Cluster A	Cluster B			Cluster C
Increased in ND	Increased in ND	Unchanged	Increased in KD	Increased in KD
3.Methylhistidine ***	4.Hydroxyproline ***	2.amino adipic.acid	Glycerol	Behenic.acid
3.phenylpropionic.acid ***	Alanine *	2.hydroxyglutaric.acid	Glycerol.3.phosphate	Benzoic.acid
5.aminovaleric.acid ***	Aminocaproic.acid *	2.oxoglutaric.acid	Glycylglycine	3.Methylglutaryl.carnitine ***
Acetylcholine ***	Creatinine *	2.hydroxy.3.methyl.butyric.acid	Tauro.alpha.muricholic/	3.hydroxybutyric.acid ***
Adenosine.2.deoxyguanosine **	Cytosine *	Hexacosanoic.acid	Tauro.beta.muricholic.acid/	Betaine. Valine
Allantoin ***	Deoxycholic.acid **	2.oxovaleric.acid	Tauro.omega.muricholic.acid	Acetate ***
Alpha.muricholic.acid ***	Docosadienoic.acid ***	Hexadecadienoic.acid	Caprylic.acid	Acetyl.carnitine ***
Beta.muricholic.acid ***	Docosenoic.acid *	Homoserine	Taurochenodeoxycholic.acid	Carnitine.C10.0 ***
Chenodeoxycholic.acid ***	Eicosenoic.acid **	Homovanillic.acid	Taurocholic.acid	Carnitine.C10.1 ***
Cholic.acid ***	Erythritol *	Alpha.tocopherol	Taurodeoxycholic.acid	Glucose.6.phosphate.Fructose.6.phosphate
Creatine ***	GABA *	Arabinose	Tauoursodeoxycholic.acid	Carnitine.C12.0 ***
Desaminotyrosine ***	Glycerophosphorylcholine **	Arachidonic.acid	Hydroxydodecanoic.acid	Glucuronic.acid.Galacturonic.acid
Docosahexaenoic.acid *	Glycine **	Arginine	Ketoisocaproic.acid	Glutathione
Docosapentaenoic.acid ***	Heptadecatrienoic.acid *	Ascorbic.acid	Ketoisovaleric.acid	Inositol
Docosatetraenoic.acid ***	Heptadecenoic.acid ***	Asparagine	Trimethyllysine	Isocaproic.acid
Docosatrienoic.acid ***	Histidine ***	Aspartic.acid	Tryptophan	Lauric.acid
Eicosadienoic.acid ***	Hypotaurine *	Azelaic.acid	Kynurenic.acid	N.acetylglutamic.acid
Eicosapentaenoic.acid ***	Indole.3.lactic.acid **	Beta.alanine	Tyrosine	N.acetylglutamine
Eicosatrienoic.acid ***	Isoleucine **	Butanoic.acid	UMP	Carnitine.C14.1.OH ***
Ferulic.acid ***	Leucine *	Caproic.acid	Lactic.acid	N1.acetylspermidine
Hippuric.acid ***	Linolenic.acid *	Carnitine	Linoleic.acid	Carnitine.C14.2 ***
Hyodeoxycholic.acid ***	Lysine ***	Carnitine.C3.0	Methylmalonic.acid	NAAG
Indole.3.acrylic.acid ***	Methionine ***	Carnitine.C4.0	Methylsuccinic.acid	Orotic.acid
Indole.3.aldehyde **	N6.acetyllysine.N2.acetyllysine ***	Carnitine.C5.0	Myristic.acid	Putrescine
Indole.3.propionic.acid ***	Nicotinic.acid **	Cholesterol	Nonanoic.acid	Ribose.Ribulose
Omega.muricholic.acid ***	Nonadecenoic.acid ***	Citrulline	Octadecanoic.acid	Succinic.acid
Palmitoleic.acid ***	Oleic.acid.Elaidic.acid ***	Cytidine	O.phosphoethanolamine	Carnitine.C8.0 ***
PCae.14.0 ***	PCae.16.0 ***	Dimethylarginine	Ornithine	Carnitine.C8.1 ***
PCae.15.0 ***	PCae.18.0 **	Docosanedioic.acid	Palmitic.acid	Citric.acid.Isocitric.acid **
PCae.16.1 ***	PCae.18.1 ***	Dodecanedioic.acid	Pantothenic.acid	Dimethylglycine ***
PCae.17.0 ***	PCae.18.3 *	Dodecanoic.acid	Phosphocreatine	Fumaric.acid ***
PCae.20.0 ***	PCae.20.4 **	Fructose	Nonanoic.acid	Hexanoylglycine
PCae.20.1 ***	Pentadecanoic.acid **	Galactitol.Sorbitol.Mannitol	O.phosphoethanolamine	Hypoxanthine **
Cae.20.2 ***	Phenylalanine **	Galactose.Glucose.Mannose	Ornithine	Lactose ***
PCae.20.3 ***	Pyruvic.acid ***	Gamma.glutamylleucine	PCae.18.2.	Maltose.Trehalose **
PCae.20.5 ***	Sphingosine.1.phosphate *	Gamma.glutamyllysine	PCae.22.6.	Raffinose ***
PCae.22.4 ***	Stearidonic.acid **	Gamma.glutamylthreonine	Pentadecenoic.acid	Ribose.5.phosphate ***
PCee.16.0 ***	Taurine *	Gamma.glutamyltryptophan	Phosphocreatine	Ribulose.5.phosphate ***
Proline.betaine ***	Threonine ***	Gamma.glutamyltyrosine	Phosphoric.acid	Sucrose ***
Tetracosenoic.acid ***	Valine **	Glutamic.acid	Proline	Xanthine **
Urea ***		Glutamine	Sebacic.acid	2.hydroxybutyric.acid **
Ursodeoxycholic.acid ***		Glutaryl.carnitine	Serine	3.Methylglutaryl.carnitine ***
Xylose ***		Glyceric.acid	Shikimic.acid	3.hydroxybutyric.acid ***

ND= Normal Diet

KD = Ketogenic Diet

**Table 1. List of metabolites differentially monitored in plasma of ketogenic diet-fed animals versus normal diet -fed littermates.**

This table refers to the non-supervised hierarchical clustering of metabolites of C57BL/6 mice fed normal diet (ND) versus ketogenic diet (KD) in plasma (Data from Figure 2B) Metabolites increased in ND are in Cluster A (in green, left column), metabolites increased in KD are in Cluster C (in red, right column) Cluster B are metabolites with few modification or unchanged (in black, middle column).